Hyperglycemic clamp and oral glucose tolerance test for 3-year prediction of clinical onset in persistently autoantibody-positive offspring and siblings of type 1 diabetic patients

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Context and Objective: In preparation of future prevention trials, we aimed to identify predictors of 3-year diabetes onset among OGTT- and hyperglycemic clamp-derived metabolic markers in persistently islet autoantibody positive (autoAb$^+$) offspring and siblings of patients with type 1 diabetes (T1D).

Design: Registry-based study.

Setting: Functional tests were performed in hospital setting.

Participants: Persistently autoAb$^+$ first-degree relatives of patients with T1D (n=81; age 5–39 years).

Main outcome measures: We assessed 3-year predictive ability of OGTT- and clamp-derived markers using receiver operating characteristics (ROC) and Cox regression analysis. Area under the curve of clamp-derived first-phase C-peptide release ($AUC_{5-10min}$; min 5–10) was determined in all relatives and second-phase release ($AUC_{120-150min}$; min 120–150) in those aged 12–39 years (n=62).

Results: Overall, the predictive ability of $AUC_{5-10min}$ was better than that of peak C-peptide, the best predictor among OGTT-derived parameters (ROC-AUC [95%CI]: 0.89 [0.80–0.98] vs. 0.81 [0.70–0.93]). Fasting blood glucose (FBG) and $AUC_{5-10min}$ provided the best combination of markers for prediction of diabetes within three years; (ROC-AUC [95%CI]: 0.92 [0.84–1.00]). In multivariate Cox regression analysis, $AUC_{5-10min}$ (P=.001) was the strongest independent predictor and interacted significantly with all tested OGTT-derived parameters. $AUC_{5-10min}$ below percentile 10

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Abbreviations: 95%CI 95% confidence interval; 2h PG OGTT-derived 2 hours post-glucose load glycemia; AutoAb(s) Autoantibody (ies); AutoAb$^+$ Positivity for IAA, GADA, IA-2A and/or ZnT8A; AIC Akaike information criterion; $AUC_{5-10min}$ First-phase C-peptide release during hyperglycemic clamp; $AUC_{120-150min}$ Second-phase C-peptide release during hyperglycemic clamp; BMI SDS Body mass index standard deviation score; FBG Fasting blood glucose; GADA Glutamate decarboxylase autoantibodies; HR High antibody-inferred risk (relatives positive for IA-2A and/or ZnT8A with at least one other antibody); IA-2A Islet antigen-2 autoantibodies; IAA Insulin autoantibodies; ICA Islet cell cytoplasmic autoantibodies; IGT Impaired glucose tolerance; IQR Interquartile range; NS Not significant; OGTT Oral glucose tolerance test; P10 Percentile 10 of the reference population; PPV Positive predictive value; ROC Receiver operating characteristics; ROC-AUC Area under the curve from the receiver operating characteristic curve; T1D Type 1 diabetes; ZnT8A Zinc transporter 8 autoantibodies

of controls was associated with 50–70% progression to T1D regardless age. Similar results were obtained for $AUC_{120-150min}$

Conclusions: Clamp-derived first-phase C-peptide release can be used as an efficient and simple screening strategy in persistently $autoAb^+$ offspring and siblings of T1D patients to predict impending diabetes.

Immunointerventions in type 1 diabetic patients have shown partial and transient efficacy in preserving residual beta cells, particularly in younger patients with short disease duration, relatively intact functional beta cell mass at diagnosis and, conceivably, some residual beta cell regenerative capacity (1, 2). These observations provide a rationale for planning similar interventions at the preclinical stage in which beta cell function is even better preserved (2–4). Given the potential adverse events associated with immune interventions (5) and the striking heterogeneity of the underlying disease process (6), the launch of such studies requires identifying individuals at very high risk to develop type 1 diabetes (T1D) in the short term (eg, 70% within 3 years). Antibody screening alone is not able to select participants with such elevated overall progression rate (7–10). Multiple genetic risk markers allow to identify a subgroup of antibody-positive ($autoAb^+$) individuals at very high risk but suffer from a low screening sensitivity (11–14). Metabolic markers such as proinsulin: C-peptide ratio and OGTT- or IVGTT-derived parameters are more sensitive and promising in this respect (15–21). In a pilot study, we have previously shown that a low first-phase C-peptide release during hyperglycemic clamp ($AUC_{5-10min}$) identifies individuals who progressed to diabetes within 2 years among relatives at high autoAb-inferred risk (multiple $autoAb^+$ and islet antigen 2 $autoAb^+$; $IA\text{-}2A^+$) (22).

Now, we expand these observations to a larger group of persistently $autoAb^+$ offspring and siblings of type 1 diabetic patients, regardless of the duration of their autoAb-positivity, the number or type of antibodies or the occurrence of dysglycemia, in order to assess whether clamp-derived C-peptide release might outperform OGTT-derived parameters in the perspective of selecting participants in secondary prevention trials with immunointervention. We therefore focused on development of diabetes within 3 years after metabolic assessment.

Materials and Methods

Study population

Persistently $autoAb^+$ offspring and siblings of patients with T1D (aged 5–39 years, n = 81) were enrolled among 2225 relatives recruited between November 1998 and July 2011 by the Belgian Diabetes Registry (10). At inclusion, demographic, personal and familial data were obtained via a questionnaire and blood samples were collected for antibody testing. Metabolic assessment consisted of OGTT and hyperglycemic clamp. Diabetes, impaired glucose tolerance (IGT) and impaired fasting glucose were diagnosed using ADA criteria (23). Six relatives had IGT at inclusion; in two of them, fasting glucose was impaired. Three of these six relatives developed diabetes within three years. Body mass index was expressed as standard deviation score (BMI SDS) in comparison to age- and sex-matched controls (24). Progression to overt diabetes was ascertained through repeated contacts with relatives, Belgian endocrinologists and pediatricians, self-reporting through yearly questionnaires and a link with the BDR patient database for new-onset patients. Follow-up started at entry and ended at diagnosis in case of diabetes or at the last blood draw. Informed consent was obtained from each relative or their legal representative. The study protocol was approved by the ethics committees of BDR and participating university hospitals and was implemented in accordance with the Helsinki declaration as revised in 2013 (http://www.wma.net/en/30publications/10policies/b3/, accessed on September first, 2014).

Oral glucose tolerance test (OGTT)

Blood samples were collected for glucose, proinsulin and C-peptide analysis before and 30, 60, 90 and 120 minutes after an oral glucose load of 1.75 mg/kg without exceeding the maximum of 75 g per os. Data on 15 minutes OGTT parameters were available in 28 persistently $autoAb^+$ relatives (35%). Peak C-peptide was defined as the highest observed C-peptide value between 0–120 minutes. C-peptide and glucose area under the curves (AUC) were calculated using the trapezium rule (22).

Hyperglycemic clamp

The test was performed 1–2 weeks after the OGTT as before (22) but without glucagon injection at min 150. Briefly, after an overnight fast, a 1.1 mol/l glucose solution was infused (Baxter, Brussels, Belgium) via the left antecubital vein at time 0. During the first 14 minutes a priming glucose dose was administered and glycemia was acutely raised to reach the desired plateau of 10 mmol/l. Thereafter, the hyperglycemic target was maintained by adjusting the glucose infusion rate upon assessment of bedside blood glucose level every 5 minutes using HemoCue® (HemoCue, Angelholm, Sweden) or Accu-check® Inform II (Roche Diagnostics, Mannheim, Germany) glucose monitors. Blood samples for C-peptide and proinsulin measurements were collected at time-points 5, 7.5 and 10 minutes to derive $AUC_{5-10min}$ and at time-points 120, 135 and 150 minutes for the second-phase C-peptide release ($AUC_{120-150min}$). C-peptide release was calculated as AUC expressed per minute. In relatives aged 5–11 years (n = 18), only the first-phase (0–10 minutes) of the clamp was performed.

The intra-individual coefficients of variation for clamp-derived beta cell function were respectively 11.8 and 11.7% for the first- and second-phase C-peptide release (22). The 10th percen-

tile (P10) of C-peptide AUC obtained from a reference population including healthy volunteers (n = 30), antibody-negative (n = 20) and transiently autoAb$^+$ relatives (n = 9) was used as cutoff value to define low $AUC_{5-10min}$ (P10 = 542 pmol.l^{-1}.min) and $AUC_{120-150min}$ (P10 = 1776 pmol.l^{-1}.min) among enrolled relatives. These three groups did not differ in clamp-derived C-peptide AUC and shared a low-risk of progression to diabetes (Supplemental Figure 1) (22). The P10 cutoff was age-independent in this control group (not shown). No development of diabetes has been reported in the reference population after a median (interquartile range; IQR) follow-up of 52 (36–132) months.

Analytical methods

Glucose was assessed with a glucose oxidase method (Vitros 950IC or 5.1 FS Ortho Clinical Diagnostics, Rochester, NY, USA), C-peptide and proinsulin by time-resolved fluorescence immunoassay (25). Between-assay coefficients of variation at low and intermediate levels were 2.4 and 1.3%, respectively for C-peptide; and 8.8 and 5.5%, respectively for proinsulin. Because of the high (100%) cross-reactivity between C-peptide and proinsulin assays, free C-peptide levels were obtained by subtracting the proinsulin concentration from the total C-peptide results (22). Diabetes-associated autoAbs against insulin (IAA) (26), glutamate decarboxylase (GADA) (27), islet antigen-2 (IA-2A) (28) and zinc transporter 8 (ZnT8A) (29) were analyzed by liquid-phase radiobinding assay (30). The radiolabeled antigen for determination of ZnT8A was obtained by in vitro transcription/translation of the dimeric CW-CR ZnT8A cDNA construct incorporating the carboxyterminal cytosolic domains (aa268–389) of both the Arg325 (CR) and Trp325 (CW) allelic variants. Results were expressed as % binding of added tracer (10 000 cpm / 50μl for GADA, IA2A and ZnT8A; 24 000 cpm/ 50 μl for IAA). Between-day coefficients of variation obtained from serum pools with antibody levels within the normal range and within the moderately elevated range were, respectively, 35% (0.3% tracer binding) and 12% (6.9% tracer binding) for IAA, 12% (2.1% tracer binding) and 10% (7.1% tracer binding) for GADA, 18% (0.3% tracer binding) and 9% (2.3% tracer binding) for IA-2A and 21% (0.7% tracer binding) and 6% (3.9% tracer binding) for ZnT8A. Diagnostic sensitivity and specificity were, respectively, 64% and 100% for GADA, 48% and 98% for IAA, 72% and 99% for IA-2A and 50% and 98% for ZnT8A (CRCW) in the 2012 Islet Autoantibody Standardization Program (IASP). Cutoff values for autoAb-positivity were determined as the 99th percentile of autoAb levels in 761 nondiabetic controls; this amounted to $\geq$ 0.6% tracer binding for IAA, $\geq$ 2.6% for GADA, $\geq$ 0.44% for IA-2A. Because ZnT8A levels tended to decrease with age in control subjects, cutoff values were calculated separately for the age groups 0–14 years ($\geq$ 1.28%) and 15–39 years ($\geq$ 1.02%) (30).

Statistical methods

Proportions were compared using χ^2 test, with Yates' correction or Fischer's exact test whereas Mann-Witney U or Kruskal-Wallis test was used for continuous data. To identify rapid progressors to diabetes, follow-up was truncated at 3 years. ROC analysis was used to compare diagnostic performance of metabolic and hormonal parameters individually or in combination. AUC of the ROC curve (ROC-AUC) with 95% CI, diagnostic sensitivity and diagnostic specificity were calculated (31). The cut-off point for optimal sensitivity and specificity was identified using the Youden index (32). The goodness of fit of the models was tested using Akaike information criterion (AIC) which represents an estimate of information not explained by the model. We selected the most predictive combination of OGTT-derived parameters based on ROC-AUC and investigated the most performant model in combination with clamp-derived markers. Independent predictors of diabetes onset derived from OGTT and hyperglycemic clamp, and their interactions were assessed using Cox proportional hazard models. Diabetes-free survival was assessed using Kaplan-Meier analysis with log-rank test. Statistical analyses were performed two-tailed by Stata 12 (StataCorp, Texas, USA). A P value < 0.05 or $< 0.05/k$ in case of k comparisons was considered statistically significant. GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) was used for the figures.

Results

Baseline characteristics of 3-year progressors vs. nonprogressors

Eighty-one persistently autoAb$^+$ offspring or siblings (age 5–39 years) of patients with T1D were enrolled. During follow-up (median [interquartile range; IQR]: 20 [12–28] months), 14 (17%) relatives (including three relatives with baseline dysglycemia) developed diabetes within three years. All three relatives with baseline dysglycemia carried a susceptible HLA-DQ genotype and at least two autoAbs. Two of them had a low first-phase C-peptide release during baseline clamp and developed diabetes within one year; the third with a borderline low value progressed after 34 months. Among the dysglycemic relatives who did not progress within three years, two single autoAb$^+$ relatives without susceptible HLA-DQ genotype showed a normal C-peptide response at baseline, despite occasional dysglycemia. A third multiple autoAb$^+$ relative with susceptible HLA-DQ genotype and recurrent dysglycemia progressed only after 83.6 months, despite a borderline low $AUC_{5-10min}$. This person was lean and had a high insulin-sensitivity as judged from repeatedly low HOMA2-IR values (0.4–0.5).

Although the vast majority of autoAb$^+$ relatives in our cohort will develop diabetes within 15 years (multiple autoAbs: 77% [95%CI: 67%–87%] under age 40 years [n = 169]; 81% [95%CI: 69%–94%] below 10 years [n = 88]; $\geq$ 1autoAb: 52% [95%CI: 44%–60%] under age 40 years [n = 390]; 74% [59%–89%] under age 10 years [n = 149]); unpublished data from Belgian Diabetes Registry) in line with other reports (33), relatives who did develop diabetes more than three years after baseline were termed 'nonprogressors' in this study. Rapid progression (within three years) indicates rapid development of T1D after metabolic testing and does not necessarily imply rapidly progressing beta cell loss. Within the whole group of

relatives, 73% (49/67) completed the three year follow-up. Age, BMI-SDS, gender and HbA1c did not differ between progressors and nonprogressors (Table 1). None of the offspring of diabetic mothers developed diabetes within three years ($P = .034$). Compared with nonprogressors, rapid progressors tended to have higher FBG and a higher prevalence of ZnT8A ($P = .007$) and multiple autoAbs ($P = .008$), particularly if one of them was IA-2A or ZnT8A ($P = .004$) (10, 11). They did not differ in fasting insulin or C-peptide levels.

Except for 2h postglucose load glycemia (2h PG), OGTT-derived parameters were significantly altered in progressors compared to nonprogressors (FBG: $P = .009$; peak C-peptide: $P < .001$; glucose AUC: $P = .015$; AUC C-peptide: $P = .003$ vs. non progressors). Similarly, clamp-derived $AUC_{5–10min}$ and $AUC_{120–150min}$ C-peptide releasewere both lower in progressors ($P < .001$). The latter also had more often $AUC_{5–10min}$ or $AUC_{120–150min}$ below the P10 of the control population ($P \leq .002$, Table 1).

ROC curves and Cox regression analysis

In the age group 5–39 years, analysis of OGTT-derived markers for participants without missing data revealed that peak C-peptide performed better than all other parameters (AUC: 0.81, 95%CI: 0.70–0.93) for prediction of 3-year diabetes onset in the study population. However, considering all metabolic parameters individually, we obtained the highest ROC-AUC with $AUC_{5–10min}$ (ROC-AUC: 0.89; 95%CI: 0.80–0.98) which also yielded the lowest AIC (Table 2). Considered together, peak C-peptide and glucose AUC performed better than any other combinations of two OGTT-derived markers (ROC-AUC: 0.86, 95%CI: 0.77–0.98) and performance was only slightly improved by introducing one, two or even three additional markers. When combining clamp-derived $AUC_{5–10min}$ with the different individual OGTT-derived parameters, FBG and $AUC_{5–10min}$ was the best combination of two parameters (not shown) compared with the best combinations of two, three, four or even all OGTT-derived markers combined with $AUC_{5–10min}$ (Table 2).

Table 1. Baseline characteristics of the study population

		Relatives according to 3-year outcome (5–39 yr)		
	All relatives	**Non-progressors**	**Progressors**	***P* value**
Characteristics	**(*n* = 81)**	**(*n* = 67)**	**(*n* = 14)**	
Age, years	17 (12–25)	17 (12–26)	19 (10–23)	.871
BMI SDS	0.34 (-0.43 – 1.09)	0.35 (-0.29 – 1.06)	−0.21 (-1.09 – 0.65)	.193
Gender, M/F (ratio)	48/33 (1.5)	40/27 (1.5)	8/6 (1.3)	.859
HbA1c, %	5.3 (4.9–5.4)	5.3 (4.9–5.4)	5.3 (5.2–5.6)	.394
HbA1c, mmol/mol	34 (30–36)	34 (30–36)	34 (33–38)	
Relationship with proband				
Sibling, n (%)	38 (47)	32 (48)	6 (43)	.738
Offspring father, n (%)	26 (32)	18 (27)	8 (57)	0.055
Offspring mother, n (%)	17 (21)	17 (25)	0 (0)	0.034
Antibodies				
IAA, GADA, IA-2A, ZnT8A, %	31/89/54/53	31/88/49[a]/46[b]	29/93/79[a]/86[b]	[a] 0.045 [b] 0.007
≥ 2 autoAb$^+$, n (%)	50 (62)	37 (55)	13 (93)	0.008
HR relatives[c], n (%)	47 (58)	34 (51)	13 (93)	0.004
OGTT data				
Fasting blood glucose, mmol/liter	4.5 (4.2–4.9)	4.4 (4.1–4.8)	4.9 (4.4–5.3)	0.009
2 h plasma glucose, mmol/liter	5.4 (4.6–6.2)	5.3 (3.9–6.2)	5.7 (5.2–7.2)	0.088
Peak C-peptide, nmol/liter	2.4 (1.8–2.7)	2.5 (1.9–2.8)	1.7 (1.5–2.1)	<0.001
Glucose AUC, mmol/liter	6.6 (5.6–7.7)	7.5 (6.6–8.3)	6.4 (5.4–7.4)	0.015
C-peptide AUC, nmol.l^{-1}·min	1.6 (1.3–2.0)	1.8 (1.4–2.2)	1.2 (1.1–1.4)	0.003
Clamp data				
C-peptide $AUC_{5–10\ min}$, pmol.l^{-1}·min	802 (585–1009)	888 (696–1084)	411 (335–590)	<0.001
C-peptide $AUC_{120–150\ min}$, pmol.l^{-1}·min	2405 (1747–3105)	2528 (2199–3182)	1327 (950–1794)	<0.001
C-peptide $AUC_{5–10\ min}$, n (%) <P10	17 (21)	8 (12)	9 (64)	<0.001
C-peptide $AUC_{120–150\ min}$, n (%) <P10[d]	16 (26)	9 (17)	7 (70)	0.002
Follow-up time, months	28 (18–51)	34 (22–62)	20 (12–28)	0.004

BMI SDS: body mass index standard deviation score; HbA1c: glycated hemoglobin; NS: not significant; AUC: area under the curve; P10: 10th percentile of the control population; IAA: insulin autoantibodies; GADA: glutamate decarboxylase autoantibodies; IA-2A: islet antigen 2 autoantibodies; ZnT8A: zinc transporter 8 autoantibodies; autoAb$^+$: positivity for IAA, GADA, IA-2A and/or ZnT8A; [c] High antibody-inferred risk (HR) relatives defined as persistent positivity for IA-2A and/or ZnT8A with at least 1 other autoAb; [d]analysis restricted to the subset of relatives aged 12–39 yr ($n = 63$) with both first- and second-phase C-peptide AUC (10 progressors and 53 non-progressors); data are count (proportion) or median (interquartile range) unless otherwise stated, threshold for significance $P < 0.005/25$ or $P < 0.002$ (Bonferroni correction).

Table 2. Receiver operating characteristic analysis in relatives aged 5–39 yr

Parameters	AUC (95%CI)	AIC	Sensitivity (%)	Specificity (%)
OGTT-derived parameters				
Fasting blood glucose, mmol.l^{-1}	0.70 (0.53–0.87)	66.9	69	68
2 h PG, mmol.l^{-1}	0.66 (0.48–0.84)	68.8	46	85
Glucose AUC, mmol.l^{-1}·min	0.72 (0.57–0.86)	66.8	85	56
C-peptide AUC, nmol.l^{-1}·min	0.77 (0.63–0.91)	62.7	77	76
Peak C-peptide, nmol.l^{-1}	0.81 (0.70–0.93)	59.6	69	87
Hyperglycemic clamp-derived marker [a]				
C-peptide $AUC_{5-10\ min}$, pmol.l^{-1}·min	0.89 (0.80–0.98)	48.1	92	76
Combinations of OGTT-derived markers				
Best combination of two markers: *peak C-peptide+ glucose AUC*	0.86 (0.77–0.98)	55.0	84	77
Best combination of three markers: *FBG+ peak C-peptide + glucose AUC*	0.88 (0.77–0.99)	53.2	77	89
Best combination of four markers: *FBG+ peak C-peptide + glucose AUC+ C-peptide AUC*	0.88 (0.77–0.99)	55.0	69	97
All OGTT-derived markers	0.88 (0.77–0.99)	57.0	69	97
Combined OGTT markers + clamp-derived markers				
FBG + C-peptide $AUC_{5-10\ min}$ [b]	0.92 (0.84–1.00)	42.4	92	84
Best combination of two markers + C-peptide $AUC_{5-10\ min}$	0.93 (0.85–1.00)	46.1	85	94
Best combination of three markers + C-peptide $AUC_{5-10\ min}$	0.93 (0.85–1.00)	44.0	92	82
Best combination of four markers + C-peptide $AUC_{5-10\ min}$	0.93 (0.85–1.00)	45.9	92	84
All OGTT-derived markers + C-peptide $AUC_{5-10\ min}$	0.93 (0.86–1.00)	47.8	92	84

AUC: area under the curve; 95%CI: 95% confidence interval; OGTT: oral glucose tolerance test; FBG: fasting blood glucose; 2 h PG: two-hour post-glucose load glycemia during OGTT; C-peptide $AUC_{5-10\ min}$: C-peptide release during the first-phase (5–10 min) of hyperglycemic clamp; C-peptide $AUC_{120-150\ min}$: C-peptide release during the second-phase (120–150 min) of hyperglycemic clamp, calculations were done in 75 study participants with complete data among whom 13 developed diabetes within 3 yr; [a] second-phase of hyperglycemic clamp was performed only in relatives aged 12–39 yr (see Supplemental Table 1); [b] best combination of a single OGTT-derived marker and $AUC_{5-10\ min}$. Data resulted from the analysis of 13 progressors among 75 relatives with complete dataset.

FBG and $AUC_{5-10min}$ was associated with the lowest AIC and a similar ROC-AUC (0.92, 95%CI: 0.84–1.00).Individually, FBG and $AUC_{5-10min}$ achieved a 50 and 64% positive predictive value (PPV) respectively, while their combination yielded 89% PPV (not shown). We obtained similar findings in the age group 12–39 years, both for $AUC_{5-10min}$ and $AUC_{120-150min}$ (Supplemental Table 1).

In univariate Cox regression analysis, all metabolic parameters derived from OGTT or hyperglycemic clamp were significantly associated with diabetes development within 3 years (Table 3). However, $AUC_{5-10min}$ outperformed each of the OGTT-derived parameters in a two-by-two multivariate analysis. Moreover, we identified a significant interaction between all OGTT-derived functional markers and $AUC_{5-10min}$ (Table 3). $AUC_{5-10min}$ also outperformed the presence of a high-risk autoAb profile in two-by-two multivariate analysis ($P < .001$ and $P = .080$ respectively; not shown).

C-peptide levels at min 15 during OGTT were available in 35% of the autoAb$^+$ relatives, but incremental C-peptide/glucose responses were not significantly correlated with $AUC_{5-10min}$ ($R^2 = 0.111$; $P = .083$; not shown).

Kaplan-Meier survival analysis based on C-peptide release during hyperglycemic clamp

In the relatives aged 12–39 years (n = 62) who underwent a complete hyperglycemic clamp, clamp-derived C-peptide release stratified progression to diabetes. Low C-peptide during at least one clamp phase (<P10) was associated with approximately 50% 3-year progression to diabetes, both before (Figure 1A) and after (Figure 1B) exclusion of relatives with dysglycemia at baseline (both $P = .001$ vs. groups with normal $AUC_{5-10min}$ and $AUC_{120-150min}$). When only considering first-phase C-peptide release in the same age group, more than 70% absolute risk of diabetes within 3 years was observed in relatives with $AUC_{5-10min}$ <P10, both in all relatives aged 12–39 years (Figure 1C) and after exclusion of individuals with baseline dysglycemia (Figure 1D, both $P < .001$ vs. groups with normal $AUC_{5-10min}$). Similar significant differences, albeit with somewhat lower overall 3-year progression rate in case of $AUC_{5-10min}$ <P10, were observed when the entire group of relatives aged 5–39 years (n = 81) was considered before (Figure 1E) or after (Figure 1F) exclusion of initially dysglycemic individuals (55% and 56% respectively; in both instances $P < .001$ vs. groups with normal $AUC_{5-10min}$). Most relatives who progressed to diabetes within three years had at least two autoAbs (13/14; 93%) When considering only multiple autoAb$^+$ relatives, those with low clamp-derived first-phase C-peptide release (<P10) also progressed faster to diabetes (5–39 years: $P = .027$ vs $AUC_{5-10min}$ >P10; 12–39 years: $P = .005$). Similar results were obtained in individuals with particularly high antibody-inferred risk (IA-2A$^+$ or ZnT8A$^+$ plus at least one other positive molecular autoAb (10) (5–39 years: $P = .015$; 12–39 years: $P = .007$) (Supplemental Figure 2 showing 84 months follow-up).

Table 3. Cox regression analysis assessing independent diabetes prediction ability of OGTT-derived parameters and first-phase C-peptide release during hyperglycemic clamp and their possible interactions

	Univariate analysis		Multivariate analysis	
Covariates	**HR (95%CI)**	***P*-value**	**HR (95%CI)**	***P*-value**
Model 1A				
FBG	4.166 (1.310–13.255)	0.016	6.382 (1.829–22.265)	0.004
$AUC_{5–10\ min}$	0.995 (0.992–0.998)	0.001	0.994 (0.992–0.997)	<0.001
Model 1B				
FBG *$AUC_{5–10\ min}$	0.999 (0.999–1.000)	0.002		
Model 2A				
2hPG	1.521 (1.081–2.141)	0.016	-	NS
$AUC_{5–10\ min}$	0.995 (0.992–0.998)	0.001	0.994 (0.991–0.997)	<0.001
Model 2B				
2hPG *$AUC_{5–10\ min}$	0.999 (0.999–1.000)	0.009		
Model 3A				
Glucose AUC	1.366 (1.019–1.832)	0.037	1.662 (1.060–2.607)	0.027
$AUC_{5–10\ min}$	0.995 (0.992–0.998)	0.001	0.994 (0.991–0.998)	0.001
Model 3B				
Glucose AUC *$AUC_{5–10\ min}$	1.000 (0.999–1.000)	0.011		
Model 4A				
Peak C-peptide	0.214 (0.067–0.683)	0.009	-	NS
$AUC_{5–10\ min}$	0.995 (0.992–0.998)	0.001	0.995 (0.992–0.998)	0.001
Model 4B				
Peak C-peptide *$AUC_{5–10\ min}$	0.998 (0.997–0.999)	0.002		
Model 5A				
C-peptide AUC	0.998 (0.997–1.000)	0.020	-	NS
$AUC_{5–10\ min}$	0.995 (0.992–0.998)	0.001	0.995 (0.992–0.998)	0.001
Model 5B				
C-peptide AUC *$AUC_{5–10\ min}$	1.000 (1.000–1.000)	0.003		

HR: hazard ratio; 95%CI: 95% confidence interval; FBG: fasting blood glucose; $AUC_{5–10\ min}$: C-peptide release during the first-phase (5–10 min) of hyperglycemic clamp; 2 h PG: two-hour post-glucose load glycemia during OGTT; AUC: area under the curve; NS: not significant.

After stratification of the study population according to FBG tertiles, a low first-phase C-peptide release during clamp conferred a higher 3-year risk of diabetes (median [95%CI]: 83% [54–100]) to relatives in the upper tertile (Figure 2C) than to those in the middle or lower tertiles (Figure 2A and 2B). We observed no difference in progression rate according to $AUC_{5–10min}$ after stratification according to tertiles of other OGTT-derived parameters (not shown). Tertiles of C-peptide release, but not 2h PG, stratified diabetes risk, both in all $autoAb^+$ relatives and in those with high autoAb-inferred risk. $AUC_{5–10min}$ performed better than OGTT peak C-peptide in this respect (Supplemental Figure 3). Although the overall progression rate to diabetes was higher in relatives with a high-risk autoAb profile than in all $autoAb^+$ relatives, a low $AUC_{5–10min}$ (<P10 of controls) was associated with equally fast progression to diabetes in both groups (Supplemental Figure 4).

Discussion

In this expanded series of persistently $autoAb^+$ siblings and offspring of type 1 diabetic patients we confirmed that individuals who progressed to diabetes within three years from metabolic testing had significantly lower $AUC_{5–10min}$ and/or $AUC_{120–150min}$ during hyperglycemic clamp than relatives who did not - or only slowly - progress to clinical onset (22). Consistent with other reports (33), most rapid progressors to T1D had at least two positive autoAbs, often comprising IA-2A and/or ZnT8A (10, 11). The major finding of this study is that hyperglycemic clamp-derived functional markers ($AUC_{5–10min}$ and $AUC_{120–150min}$) outperformed the most informative OGTT-derived parameters to identify relatives with impending diabetes regardless of the autoAb-inferred risk profile. The 3-year risk conferred by $AUC_{5–10min}$ <P10 of controls amounted to 50%–70% in the overall $autoAb^+$ study population, reaching up to 83% if FBG was in the upper tertile. These results suggest that a 10 minutes hyperglycemic clamp test, determining only $AUC_{5–10min}$ C-peptide, is sufficient to identify most rapid progressors and is applicable and well accepted over a wide age range (22). Our results were also valid when only relatives with a high autoAb-inferred risk were considered, indicating that low clamp-derived C-peptide release is not a surrogate for multiple autoAbs, but an important additional marker to identify, among individuals who will almost all develop diabetes within 15 years (33, 34), those who are closest to clinical onset.

Focusing on 3-year progression to diabetes – an interval relevant for prevention studies – constitutes a strength of this study. Participants were enrolled based on positivity for molecular antibodies without considering cytoplasmic islet cell antibodies (ICA). The latter test yields only semi-quantitative results and is not entirely independent of the molecular antibody markers (10). The relatively small number of progressors to diabetes within three years is a major limitation, but we restricted the number of covariates in multivariate analysis by assessing two-by-two models to comply with the event–to-variable ratio of Vittinghoff (35). We could therefore not adjust for clinical parameters (age, BMI, relationship to the proband) but this does not invalidate the comparison with OGTT-derived parameters. Moreover, our survival analyses indicate that our prediction model is equally valid above age 12 years. Our study population was more homogeneous than that of certain other programs since we only included persistently autoAb$^+$ siblings or offspring, but not second-degree relatives (19). Unlike certain other cohorts (eg, DAISY, TEDDY, BABYDIAB) (9), relatives were not followed from birth on. However, we do not believe this to

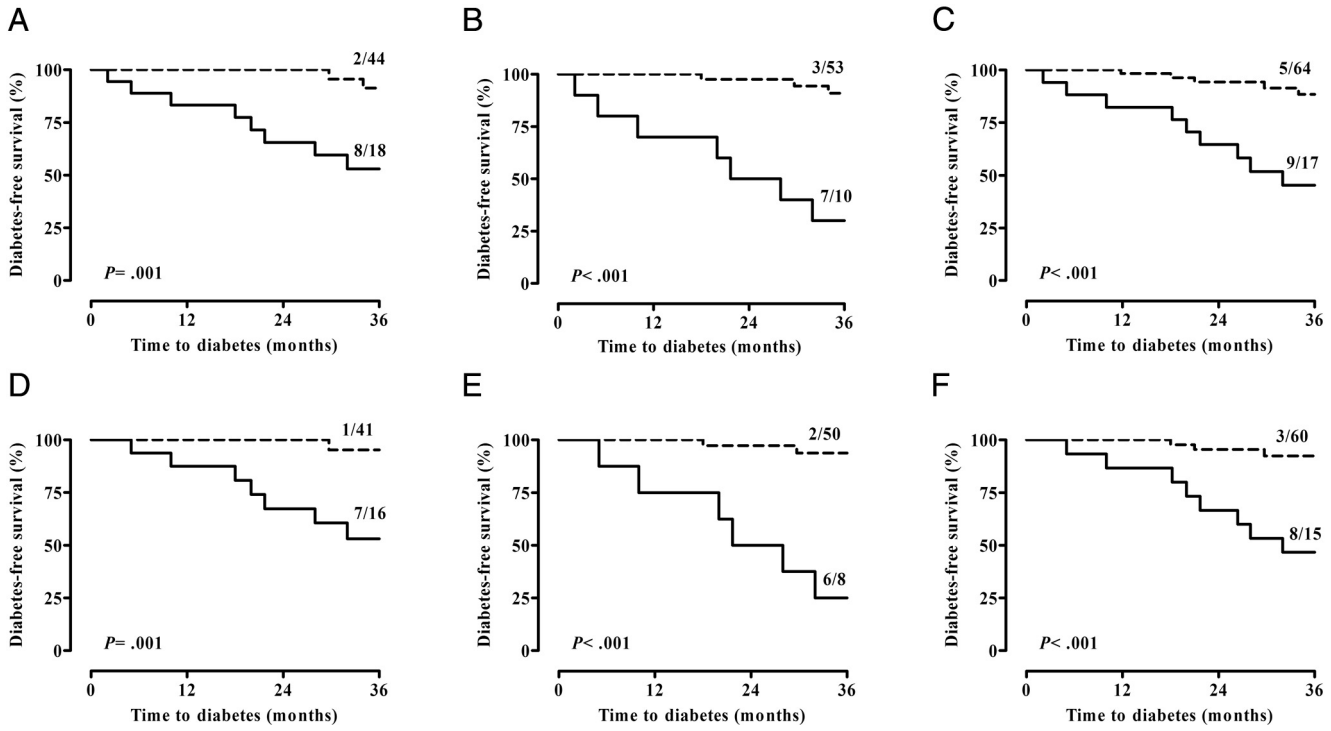

Figure 1. Diabetes-free survival after stratification according to clamp-derived C-peptide AUC below or above percentile 10 (P10) of the control population (see methods) before (panels A, B and C) and after (panels D, E and F) exclusion of relatives with dysglycemia. Panels A (n = 62) and D (n = 57): $AUC_{5–10\ minutes}$ and/or $AUC_{120–150\ minutes}$ <P10 (solid line) and $AUC_{5–10\ minutes}$ and $AUC_{120–150\ minutes}$ ≥P10 (broken line) in the age group 12–39 years; panels B (n = 63) and E (n = 57): $AUC_{5–10\ minutes}$ <P10 (solid line) and $AUC_{5–10\ minutes}$ ≥P10 (broken line) in the age group 12–39 years; panels C (n = 81) and F (n = 75): $AUC_{5–10\ minutes}$<P10 (solid line) and $AUC_{5–10\ minutes}$ ≥P10 (broken line) in the age group 5–39 years.

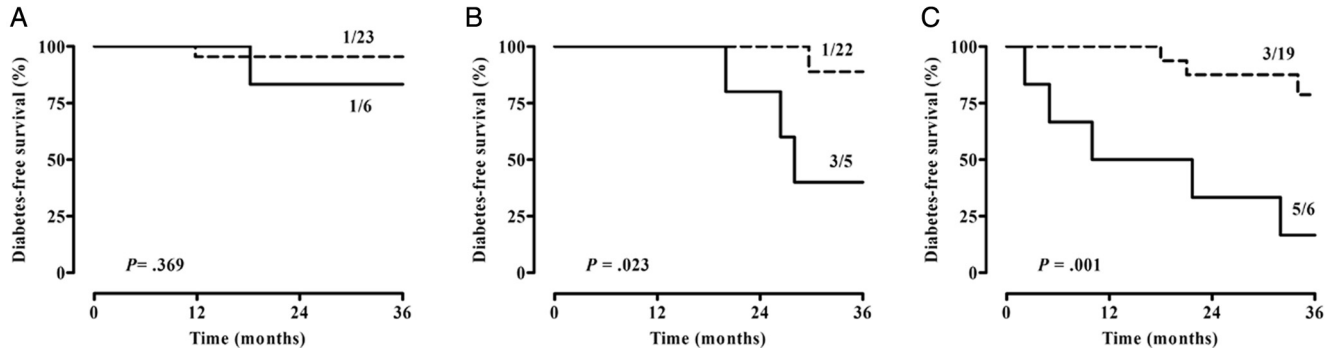

Figure 2. Diabetes-free survival in autoAb$^+$ relatives stratified according to fasting blood glucose tertiles: First-phase C-peptide release during hyperglycemic clamp below (solid line) or above (broken line) percentile 10 (P10) of the control population stratified according to the first (panel A; FBG ≤ 4.28 mmol.l^{-1}), second (panel B; 4.28 < FBG ≤ 4.78 mmol.l^{-1}) and third (panel C; FBG ≥ 4.78 mmol.l^{-1}) tertiles of fasting blood glucose. The numbers in each tertile are slightly different because there were several participants with identical glycemia levels.

be relevant, since presently inclusion in immunointervention trials is not considered under age 5 years. Finally, we could not report C-peptide concentrations 15 minutes postglucose load during OGTT which have been shown to be more informative than the value after 30 minutes (36). As we started to collect these data after this study was initiated, they are only available in 35% of the participants. At variance with a previous report (36), we did not find a significant correlation between the incremental C-peptide/glucose response 15 minutes postglucose load during OGTT, possibly related to differences in study population and C-peptide range tested (not shown). Finally, our results need to be confirmed prospectively in an independent cohort.

The DPT-1 study group recently reported that in ICA^+ individuals with dysglycemia and/or decreased insulin release during IVGTT, the combination of OGTT-derived peak C-peptide, 2h PG and C-peptide AUC improved accuracy in predicting progression to T1D (19). Moreover, IVGTT-derived measurements did not further raise the predictive ability. In contrast, our results indicate that C-peptide release under intravenous (IV) glucose stimulation during a hyperglycemic clamp – the gold standard for β-cell function (37) – is more accurately predicting rapid progression to diabetes than the selected most informative OGTT-derived parameters (2h PG, AUC glucose and peak C-peptide) (16, 19), even in individuals with a completely normal OGTT. The higher reproducibility of the clamp test as compared with IVGTT may help explain the difference between both studies (22, 38). In the age group 12–39 years, the diagnostic performance of C-peptide release under prolonged glycemic stimulation ($AUC_{120-150min}$) during a full clamp was not superior to that of $AUC_{5-10min}$ for predicting rapid progression. Assessment of $AUC_{5-10min}$ is applicable on a larger scale than the more cumbersome full clamp procedure in the age range 5–39 years, relevant for immune interventions, as it can be completed within 30 minutes and does not require adjustment of the glucose infusion rate after the priming dose. It is also more reproducible than the 10 minutes IVGTT after glucose bolus (38) and shorter than the 1–3h IVGTT for minimal model analysis (39). The introduction of an additional functional test to the standard clinical OGTT procedure, which is anyway needed to detect dysglycemia, might be considered a disadvantage and an impediment to the widespread application of such mini-clamps. However, the latter are in our experience well accepted and tolerated by relatives, also children, and their better predictive performance supports their implementation. Since a low $AUC_{5-10min}$ is also the best prognostic marker in normoglycemic relatives, a prediction strategy based on the use of these "mini-clamps" (first 10 minutes) in $autoAb^+$ individuals opens perspectives for considering using the development of dysglycemia as surrogate endpoint in prevention trials (2, 40).

Whether impairment of $AUC_{120-150min}$ during hyperglycemic clamp generally precedes the drop in $AUC_{5-10min}$ could not be determined in the present analysis of baseline clamps. If proven, this parameter could represent an earlier indicator of beta cell loss as previously suggested (22) and might become instrumental in predicting diabetes development over a longer period. An ongoing longitudinal study is investigating temporal changes in first- and second-phase C-peptide release in relation to diabetes development in $autoAb^+$ relatives. $AUC_{120-150min}$ also allows to monitor functional beta cell mass after clinical onset and was validated as predictor of therapeutic response and outcome measure in tertiary immune intervention trials and β-cell replacement therapy (2).

In conclusion, a decreased first- and second-phase C-peptide release during hyperglycemic clamp precedes clinical onset and often dysglycemia in $autoAb^+$ relatives. First-phase C-peptide release ($AUC_{5-10min}$) better discriminates rapid (ie, within three years) progressors to diabetes from slow or nonprogressors than OGTT-derived measures. A 10 minutes mini-clamp procedure is instrumental in identifying candidates of choice for secondary prevention trials with immune intervention over a wide age range among $autoAb^+$ individuals. Such trials will help define the minimal functional beta cell mass is needed for therapeutic efficacy in preT1D.

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